AT2433-A1, AT2433-A2, AT2433-B1 AND AT2433-B2 NOVEL ANTITUMOR COMPOUNDS PRODUCED BY *ACTINOMADURA MELLIAURA*

II. STRUCTURE DETERMINATION

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The chemical structures of AT2433-A1 (**2**), AT2433-A2 (**3**), AT2433-B1 (**4**) and AT2433-B2 (**5**) were elucidated by degradation and spectroscopic studies. Compounds **2**~**5** are disaccharides closely related to rebeccamycin. The aglycone in **2** and **3** was determined to be 6-*N*-methyl-11-chloro-5*H*-indolo[2,3-*a*]pyrolo[3,4-*c*]carbazole and in **4** and **5** it was determined to be 6-*N*-methyl-5*H*-indolo[2,3-*a*]pyrolo[3,4-*c*]carbazole. Compounds **2** and **4** are 4-*N*''-methyl analogs of **3** and **5**.

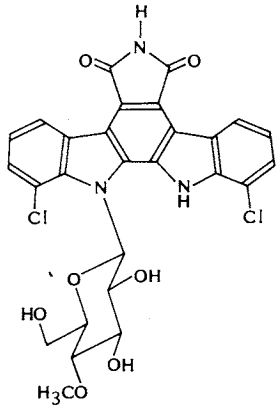
AT2433-A1, AT2433-A2, AT2433-B1 and AT2433-B2 are new antitumor antibiotics which were isolated from the fermentation broth culture *Actinomadura melliaura* ATCC 39691. These compounds are closely related to the antitumor antibiotic rebeccamycin (**1**)[1~3]. In the preceding paper[4], the taxonomy, fermentation, purification, physico-chemical, and biological properties of these compounds were described. This paper describes the structure determination of AT2433-A1, -A2, -B1 and -B2.

Based on an analysis of the spectroscopic data and degradation work, we have assigned structures **2**~**5** to AT2433-A1, -A2, -B1 and -B2, respectively (Fig. 1). Since **2** was available in the largest quantities, spectroscopic and degradation studies focused on this material. The close resemblance of **2**~**5** to rebeccamycin was initially recognized by comparison of their ^{1}H NMR, UV and IR spectra. Tabulated in Tables 1, 2 and 3, respectively, are the ^{1}H NMR, UV and ^{13}C NMR spectra for **2**~**5**. The nearly identical absorption bands in the UV and IR disclosed the presence of a common chromophore which for rebeccamycin is a 5*H*-indolo[2,3-*a*]pyrolo[3,4-*c*]carbazole[1]. The ^{1}H NMR spectra of **2**~**5** confirmed the presence of this group. There were, however, several significant differences. The down field singlet at δ 11.37, the phthalimide NH signal of rebeccamycin, was absent and the presence of a methyl singlet at δ 3.25 indicated methyl substitution on this nitrogen. Also, in the spectra of **2** and **3** an additional aromatic proton was observed at δ 7.88 and in the spectra of **4** and **5** two additional aromatic protons appeared at δ 7.86 and 8.14. This suggested that one of the chlorine atoms of the rebeccamycin chromophore was absent in compounds **2** and **3** and both chlorine atoms were absent in compounds **4** and **5**. This was confirmed by IR and MS data.

The IR spectra of **2**~**5** showed bands characteristic of hydroxyl groups and a cyclic imide at 3420, 3365, 1750 and 1962 cm^{-1}. Asymmetric substitution of the chlorine atoms in the benzene rings resulted in strong split absorption bands at 768 and 759 cm^{-1} in **2** and 765 and 755 cm^{-1} of **3**. In **4** and **5** a strong single absorption at 750 cm^{-1} was observed.

The molecular weights of compounds **2**~**5** were determined by chemical ionization (CI) or field desorption (FD)-MS. The following molecular ions were identified:

Fig. 1. Structures of rebeccamycin (**1**), AT2433-A1 (**2**), -A2 (**3**), -B1 (**4**) and -B2 (**5**).



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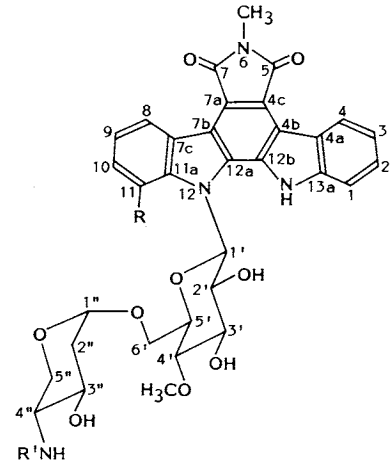


2 R=Cl R′=CH_3
3 R=Cl R′=H
4 R=H R′=CH_3
5 R=H R′=H

Table 1. ^{1}H NMR chemical shifts* and assignments for rebeccamycin (**1**), **2**, **3**, **4** and **5**.

Proton**	1	2	3	4	5
1-H	—	7.88	7.88	7.86	7.86
2-H, 10-H	7.74, 7.69	7.73	7.73	7.68	7.68
3-H, 9-H	7.45, 7.45	7.49	7.49	7.48	7.48
4-H	9.09	9.27	9.27	9.32	9.32
6-NH	11.37	—	—	—	—
13-NH	10.30	10.64	10.64	10.50	10.50
8-H	9.27	9.18	9.18	9.21	9.21
11-H	—	—	—	8.14	8.14
1′-H	6.97	6.91	6.91	6.44	6.44
2′-H	3.44	a	c	e	g
2′-OH	5.03	5.06	5.06	d	f
3′-H	3.59	a	c	e	g
3′-OH	5.45	5.48	5.48	d	f
4′-H	3.67	a	c	e	g
4′-OCH_3	3.61	3.68	3.68	3.68	3.68
5′-H	3.87	4.08	4.08	e	g
6′-H_a	3.99	4.21	4.21	e	g
6′-H_b	3.99	3.99	3.99	e	g
6′-OH	5.36	—	—	—	—
1″-H	—	5.13	5.14	d	f
2″-H_a	—	b	2.32	2.03	2.03
2″-H_b	—	1.78	1.73	1.60	1.60
3″-H	—	3.77	c	e	g
3″-OH	—	4.81	4.81	d	f
4″-H	—	3.40	c	e	g
4″-NH	—	b	2.55		
4″-NCH_3	—	2.22	—	2.19	—
5″-H_a	—	a	c	e	g
5″-H_b	—	a	c	e	g
Overlap		a 3.70~3.60 b 2.30	c 3.72~3.30	d 5.6~4.6 e 4.3~3.2	f 5.6~4.6 g 4.3~3.2

* Chemical shifts in DMSO-d_6 solution from TMS (δ) at 360 MHz.
** As indicated in Fig. 1.

2 (CI-MS)	*m/z* 679 (M+H)	$C_{34}H_{35}ClN_4O_9$
3 (FD-MS)	*m/z* 664 (M), 687 (M+Na)	$C_{33}H_{33}ClN_4O_9$
4 (FD-MS)	*m/z* 644 (M), 667 (M+Na)	$C_{34}H_{36}N_4O_9$
5 (FD-MS)	*m/z* 630 (M), 653 (M+Na)	$C_{33}H_{34}N_4O_9$

The high resolution (HR)FD-MS allowed us to determine the elemental composition and furthermore, the isotopic clusters for the molecular ions, observed for **2** and **3** indicated the presence of one chlorine atom in each of these molecules. Crucial structural information was obtained from electron impact (EI)-MS of the more volatile tetraacetate derivatives of **2**~**5**. Their volatility allowed us to record abundant molecular ions and the fragmentation pattern which indicated the sequence of the carbohydrate units and their mutual relationship (Fig. 2).

The resemblance of **2**~**5** to rebeccamycin was further reflected in their NMR spectra facilitating the signal assignment particularly of the 4-methoxy glucose and chromophoric aglycones. The presence of the 4-methoxyglucopyranoside fragment was readily apparent by inspection of the ^1H and ^{13}C NMR data of **2**. The absorption for the C-4′ methoxyl protons appeared at δ 3.68 and the C-1′ anomeric methine proton appeared at δ 6.91 in close agreement with rebeccamycin. The ^{13}C absorptions for C-1′~C-5′ and 4′-OCH_3 were also very close to those observed in rebeccamycin. The absorption for C-6′ was shifted down field 6.3 to 66.0 ppm indicating the point of further substitution.

Table 2. UV absorption maximum for **2**~**5** (λ_{max}^{MeOH} nm (ε)).

2	3	4	5
200 (30,800)	198 (30,500)	202 (29,000)	201 (30,800)
235 (39,900)	234 (38,600)	234 (41,300)	233 (41,100)
283 (34,300)	286 (33,600)	284 (33,600)	282 (33,400)
316 (45,600)	314 (43,900)	316 (46,900)	315 (46,900)
395 (3,900)	394 (3,500)	400 (4,100)	400 (4,000)

Localization of the *N*-glycoside bond at the peri position to the chlorine atom was established by comparison of the chemical shifts of the anomeric protons in the A and B congeners. The proximity of

Table 3. ^{13}C NMR chemical shifts and assignment for rebeccamycin (**1**) and AT2433-A1 (**2**).

	Chemical shift (ppm)[a]		M[b]		Chemical shift (ppm)[a]		M[b]
	1	2			1	2	
C-1[c]	116	112.1	d	C-12b	129.5	129.5	s
C-2	129.7	129.7	d	C-13a	137.0	138.1	s
C-3	121.9	121.0	d	C-1′	84.2	84.7	d
C-4	123.1	124.6	d	C-2′	72.0	72.2	d
C-4a	121.4	121.4	s	C-3′	77.5	78.8	d
C-4b	117.6	118.4	s	C-4′	79.3	78.1	d
C-4c	118.4	119.2	s	C-5′	80.3	77.5	d
C-5, C-7	170.1	169.0	s	C-6′	59.7	66.0	t
C-7a	121.4	121.3	s	C-1″	—	99.0	d
C-7b	119.2	117.6	s	C-2″	—	37.0	t
C-7c	125.3	125.3	s	C-3″	—	66.5	d
C-8	123.1	123.4	d	C-4″	—	61.7	d
C-9	122.4	122.4	d	C-5″	—	61.8	t
C-10	126.9	127.8	d	4′-OCH_3	60.0	60.1	q
C-11	116.5	116.4	s	4″-NCH_3	—	33.9	q
C-11a	137.4	140.2	s	6-CH_3	—	23.6	q
C-12a	129.5	130.0	s				

[a] Chemical shifts in DMSO-d_6 from TMS.
[b] Multiplicities (M) from off resonance decoupling.
[c] As indicated in Fig. 1.

Fig. 2. MS fragmentation patterns of the peracetates of AT2433-A1 (**2**), -A2 (**3**), -B1 (**4**) and -B2 (**5**).

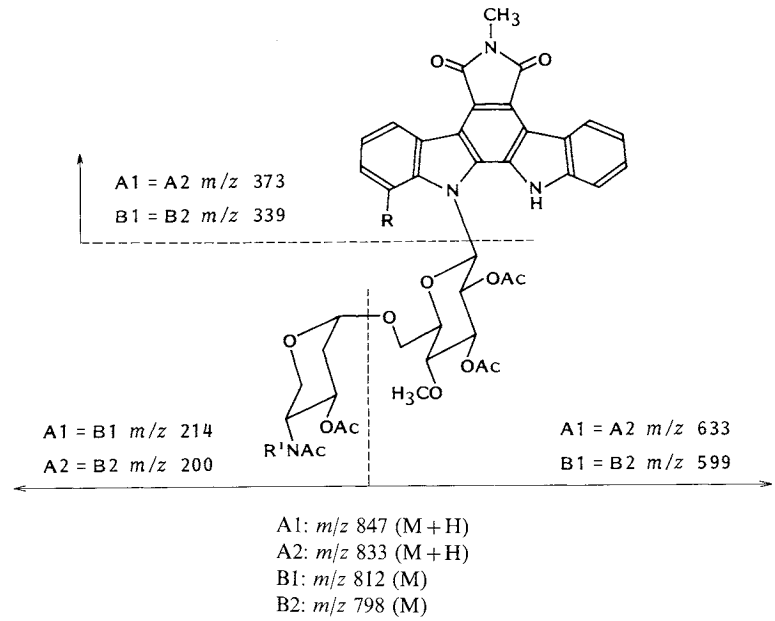
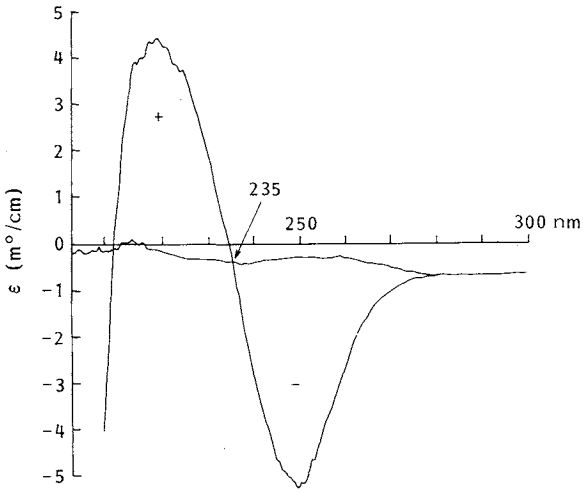
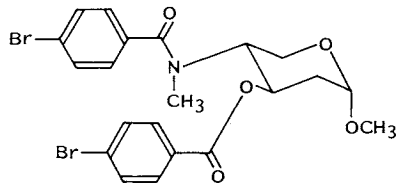


Fig. 3. Structure and CD curve of **6**.





6

Spectrum recorded in acetonitrile, sensitivity scale: 2 m°/cm, concentration 0.02 mg/ml, CD cell: d = 1 cm.

the electronegative chlorine atom to the glycosidic bond caused a strong down field shift of the anomeric protons of **2** and **3** to 6.85 ppm, comparable to 1'-H at 6.97 ppm of rebeccamycin. The anomeric protons of **4** and **5**, the dechloro congeners, are found at 6.40 ppm. This comparison readily allowed us to localize the glycosidic bond at N-12 of **2** and **3**. In addition, the shift of C-1' at 81.7 ppm was typical for a *N*-glycosidic linkage.

A chain of consecutive coupling constants ranging from 9~9.5 Hz of the sugar protons indicated the *β*-gluco configuration of the pyranose ring. The triplet of the 6'-OH proton observed in the DMSO-d_6 spectrum of **1** at 5.32 ppm was missing in **2**~**5**, further suggesting the attachment of the aminosugar

moiety at this position. The peracetylated derivatives showed negligible change of the chemical shifts of the 6′-H protons (3.99 ppm) and substantial differences (Δ ppm) for 2′-H (5.11 ppm) and 3-H (5.57 ppm) when compared to the underivatized compounds. The methoxyl at C-4′ is common to both **1** and **2**~**5** and can readily be identified from the NMR spectrum.

The structures of the novel aminosugars were elucidated by additional MS, NMR and CD studies of the methanolytic degradation products of **2**. Comparison of the ^{1}H NMR spectra of **2**~**5** indicated that the aminosugar in **4** was identical with that in **2** and that the amino sugars in **3** and **5** were the *N*-demethyl analogs of those in **2** and **4**. The product of the degradation of **2**, methyl 2,4-dideoxy-4-*N*-methylaminopentapyranoside, was separated and derivatized with 4-bromobenzoyl chloride in pyridine to yield its 3,4-di-*p*-bromobenzoate (CI-MS *m/z* 528 (M+H), two bromine atoms) which was examined by ^{1}H NMR in DMSO-d_6 at 110°C (due to the rotational barrier about the amide bond a highly complex spectrum was observed below 110°C). The assignment of the protons (Table 4) allowed us to propose the α configuration at C-1″ and an all equatorial substitution of the pyranose ring at C-3″ and C-4″. The α configuration is also present in **2**~**5** as shown by ^{1}H NMR. The CD spectrum of this derivative showed a typical split of Cotton effects (CE) at 236 mm. Since the first CE at λ_{max} 250 nm is negative the chirality of the 3,4-di-*p*-bromobenzoate is α-D-*threo* (Fig. 3)[5]. In addition the CD spectra of **2**~**5** and rebeccamycin (**1**) are superimposable indicating identical chirality among all these compounds. This establishes both the relative and absolute structures for **2**~**5** as those shown in Fig. 1.

Table 4. ^{1}H NMR chemical shift and assignment for **6**, methyl 2,4-dideoxy-4-*N*-methylaminopentapyranoside 3,4-di-*p*-bromobenzoate.

	Chemical shift (δ)[a], multiplicity (J=Hz)
1-H[b]	4.84 br s
1-OCH_3	3.36 s
2-H_a	2.30 br dd (10.0, 4.4)
2-H_b	1.82 br m
3-H	5.62 dt (10.0, 11.0)
4-H	4.40~4.20 br m
4-NCH_3	2.83 s
5-H_a	3.95 dd (10.0, 11.0)
5-H_b	3.77 dd (10.0, 5.2)
Ar (8H)	7.9~7.2 m

[a] Chemical shifts in DMSO-d_6 at 110°C from TMS.
[b] As indicated in Fig. 3.

Experimental

General

The UV spectra were recorded on Beckman Acta III Instrument in methanol. The IR spectra were measured on Beckman IR, Model 4230 as a KBr pellets. The CI and EI-MS were obtained on a Hewlett-Packard HP5985B spectrometer. The FD low and HR-MS were obtained using a Varian MAT-731 instrument. The ^{1}H and ^{13}C NMR spectra were obtained on a Bruker WM360 Instrument in DMSO-d_6. Circular dichroism was measured with Jasco 500A spectrometer in acetonitrile.

Peracetylation of **2**~**5**

The samples of **2**~**5** (**2**, 6 mg) were dissolved in *ca.* 1 ml of pyridine and 100 ml of acetic anhydride were added. The reaction mixture was kept at room temperature 16 hours. Then solvents were evaporated to dryness in vacuum and the products of acetylation were purified by flash chromatography in ethyl acetate.

Methyl 2,4-Dideoxy-4-*N*-methylamino-α-D-*threo*-pentapyranoside 3,4-Di-*p*-bromobenzoate (**6**)

A sample of **2** (25 mg) was dissolved in 1 ml of methanol and 1 ml of methanol and 1 ml of 4.5 N solution of hydrogen chloride in methanol was added. The reaction mixture was refluxed 16 hours. Approximately 80% of the starting material was methanolyzed (monitored by TLC). An extension of the reaction time led to a higher rate of by-product formation. The reaction mixture was cooled to room temperature and was neutralized with an excess of silver carbonate. The precipitate was removed by

centrifugation and the solvents were evaporated to dryness. The methyl glycoside was separated from the dry residue by flash chromatography in chloroform - methanol (5 : 1) with total yield 59%. The compound (~3.0 mg) was dissolved in 2 ml of pyridine and 10 mg of *p*-bromobenzoyl chloride was added. The reaction mixture was stirred 16 hours at 60°C. Following this the pyridine was evaporated *in vacuo* and the dry residue was extracted with hexane. Crude **6**, contained in the hexane extract, was purified by silica gel chromatography on a 20 × 20 cm plate (250 μm) using ethyl acetate - hexane (1 : 1) and eluted from the silica gel with chloroform.

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